Material Properties and Cytocompatibility of Injectable MMP Degradable Poly(lactide ethylene oxide fumarate) Hydrogel as a Carrier for Marrow Stromal Cells

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Injectable in situ crosslinkable biomaterials seeded with multipotent progenitor cells and coupled with minimally invasive arthroscopic techniques are an attractive alternative for treating irregularly shaped osteochondral defects. An in situ crosslinkable poly(lactide-*co*-ethylene oxide-*co*-fumarate) (PLEOF) macromer has been developed with ultralow molecular weight poly(L-lactide) and poly(ethylene glycol) (PEG) units linked by fumaryl unit. The PLEOF macromer was crosslinked with the MMP-13 degradable peptide sequence QPQGLAK with acrylate end-groups or the methylene bisacrylamide (BISAM) crosslinker to form enzymatically or hydrolytically degradable hydrogels, respectively. Cell viability of the peptide crosslinker was significantly higher than that of BISAM. The relatively higher molecular weight peptide crosslinker significantly affected the water content and the rate of crosslinking (e.g., sol vs gel fraction). The addition of a small fraction of a highly reactive BISAM crosslinker to the PLEOF/peptide mixture reduced the gelation time and increased the elastic modulus while retaining enzymatic degradability of the hydrogel. Bone marrow stromal (BMS) cells were encapsulated in the peptide crosslinked PLEOF hydrogel; 84% of the encapsulated cells was viable after 1 week of incubation in osteogenic media. The encapsulated BMS cells differentiated to osteoblasts and produced a mineralized matrix, as measured by ALPase activity and calcium content. The degradation rate of the hydrogel depended on the ratio of the peptide to the BISAM crosslinker, MMP-13 concentration, and incubation time. The results demonstrate that the peptide crosslinked PLEOF hydrogel with tunable degradation characteristics is potentially useful as an injectable in situ crosslinkable carrier for bone marrow stromal cells.

Introduction

An estimated 40 million Americans are affected by arthritis, a condition associated with degeneration of the involved joint surfaces,[1] and one million patients every year undergo surgery for osteoarthritis of their knee, hip, shoulder, or spine.[2] In addition to joint space narrowing, the degenerative articular cartilage changes often are associated with peripheral joint osteophytosis, subchondral bone sclerosis, and cystic bony changes.[3,4] Osteochondral autografts and allografts are currently among the options used clinically to replace traumatic osteochondral defects.[5,6] However, autografts require invasive surgical techniques to transfer good cartilage with a plug of underlying bone from a donor site or from other non-load-bearing parts of the joint, which have little contact with other surfaces, to an arthritic weight-bearing section of the joint. Allografts have the potential for disease transmission and immunologic rejection.

One approach to biological restoration of degenerated articular cartilage is the use of tissue engineering[7] strategies to transplant undifferentiated progenitor marrow stromal cells,[8] placed in a supportive carrier,[9] into the osteochondral defects.[10,11] Injectable biomaterials seeded with cells and coupled with minimally invasive arthroscopic techniques are an attractive alternative for treating irregularly shaped osteochondral defects with minimum tissue dissection and retraction.[12,13] After injection and hardening in situ, these three-dimensional hydrogel matrices guide the organization, differentiation, proliferation, and development of seeded cells into the desired tissue.[14]

Naturally based injectable hydrogels that have been investigated for this purpose include alginate,[15] fibrin,[16,17] gelatin,[18] collagen types I and II,[19,20] and hyaluronic acid.[21] Preliminary results are promising for naturally derived polymeric materials, but their low mechanical properties, their pathogenicity, and their limited availability have prompted researchers to investigate the use of synthetic hydrogels. The use of non-degradable hydrogels such as poly(ethylene oxide),[22] poly(vinyl alcohol),[23,24] and poly(acrylamide)[25] as injectable scaffolds with seeded cells is limited by their persistence within the regenerated tissue volume. Poly(lactic acid), poly(glycolic acid), and their copolymers have been used as plugs to fill osteochondral defects, but these degradable copolymers are not injectable.[26−28]

Injectable synthetic hydrogel matrices based on poly(ethylene) glycol (PEG) modified with reactive acrylate[29−31] or fumarate groups[32] have been developed for filling cartilage defects and as carriers for transplanted cells. These hydrogels can be functionalized with adhesive peptides to promote attachment and migration of bone marrow stromal (BMS) cells.[33] Although these hydrogels degrade by hydrolysis,[34] their degradation is not coordinated with BMS cell migration, proliferation, or extracellular matrix formation.[35]

Scaffolds that mimic the tissue's extracellular matrix (ECM) can be remodeled by the regenerating tissue and do not leave a residue that impedes functional adaptation.[36] For example, in the process of fetal development or wound healing, remodeling of the ECM is mediated by a number of proteolytic enzymes secreted or locally activated by the migrating cells.[37,38] In bone remodeling, matrix metalloproteinases (MMPs), secreted by the cellular components and released to the extracellular matrix,

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degrade the collagen network,[39] and MMP substrates have been used as crosslinkers to produce proteolytically degradable artificial ECMs. For example, non-degradable PEG macromers crosslinked with GPQGIWGQ bis-cystine peptide are preferentially degraded by MMP-1,[37] while non-degradable *N*-isopropylacrylamide (NIPAAm) and acrylic acid (AAc) macromers crosslinked with QPQGLAK bis-acrylate peptide are degraded by MMP-13.[40,41]

Recently, our laboratory has developed an injectable resorbable macromer that can be crosslinked in situ using a neutral redox initiation system to form a hydrogel.[42] The macromer is based on a poly(lactide-*co*-ethylene oxide-*co*-fumarate) (PLEOF) terpolymer consisting of ultralow molecular weight poly(L-lactide) (ULMW PLA) and poly(ethylene oxide) (PEG) blocks linked by unsaturated fumarate units.[43] The water content and mesh size of the hydrogel can be adjusted by the ratio of the hydrophilic PEG to hydrophobic PLA blocks and by the molecular weight of PEG.[44] The network density can be controlled by the density of fumarate groups on the terpolymer chains. The unsaturated fumarate group in the macromer can be used to covalently attach biologically active peptide sequences in the hydrogel network. The objective of this work was to determine the material properties and cytocompatibility of an in situ crosslinkable PLEOF hydrogel crosslinked with an MMP degradable peptide sequence as an injectable carrier for BMS cells. The amino acid sequence Lys-Ala-Leu-Gly-Glu-His-Lys with acrylate end-groups, synthesized in the solid phase, was used as the crosslinker. The peptide crosslinker is degraded by MMP (collagenase type III; MMP-13) secreted by migrating BMS cells.[45] The PLEOF macromer crosslinked with an MMP degradable peptide is potentially attractive as a bimodally degradable hydrogel with hydrolytic as well as enzymatic degradation modes.

Experimental Procedures

Materials. The Rink Amide NovaGel and the protected amino acids were purchased from EMD Biosciences (San Diego, CA). Reagent grade solvents for peptide synthesis and purification, *N*,*N*-diisopropylethylamine (DIEA), *N*,*N*-dimethylformamide (DMF), *N*,*N*′-diisopropylcarbodiimide (DIC), triisopropylsilane (TIPS), *N*,*N*-dimethylaminopyridine (DMAP), hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA), and acetonitrile (MeCN), were purchased from Acros Organics (Pittsburgh, PA) and used as received. Precipitation solvents diethyl ether and hexane (anhydrous grade) were purchased from VWR (Bristol, CT). Piperidine, triethylamine (TEA), ammonium persulfate (APS, electrophoresis grade), *N*,*N*′-methylenebis(acrylamide) (BISAM), triethylamine (TEA), acrylic acid, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA), and tin (II) 2-ethylhexanoate (TOC) were purchased from Aldrich (Milwaukee, WI). Fumaryl chloride (FuCl; Aldrich) was purified by distillation, and PEG (Aldrich; nominal molecular weight of 4.3 kDa) was dried by azeotropic distillation from toluene. Methylene chloride (MC; Acros Organics) was dried by distillation over calcium hydride (Aldrich). Diethylene glycol (DEG) was purchased from Acros Organics. l-LACTIDE MONOMER (LA; >99.5% PURITY BY GC) WAS PURCHASED FROM ORTEC, INC. (EASLEY, SC). ALL OTHER SOLVENTS WERE REAGENT GRADE AND USED AS RECEIVED.

Dulbecco's phosphate-buffered saline (PBS) and Dulbecco's Modified Eagle's Medium (DMEM; 4.5 g/L glucose with L-glutamine and without sodium pyruvate) were purchased from Cellgro (Mediatech; Herndon, VA). Ethylenediaminetetraacetic acid disodium salt (EDTA) and trypsin were purchased from Sigma (St. Louis, MO) and Invitrogen (Carlsbad, CA), respectively. Heat-inactivated fetal bovine serum (FBS), screened for compatibility with rat bone marrow stromal cells, was purchased from Atlas Biologicals (Fort Collins, CO). The enzyme matrix metalloproteinase-13 (collagenase CLS-3; MMP-13; 150 units/mg dw) was purchased from Worthington Biochemical (Lakewood, NJ). The Live/Dead cell viability/cytotoxicity kit was purchased from Molecular Probes (Eugene, OR).

Characterization. The chemical structure of the PLEOF macromer was characterized by a Varian Mercury-300 ^{1}H NMR (Varian, Palo Alto, CA) at ambient conditions. Pulse angle, pulse width, recycle time, acquisition time, resolution, and number of scans were 90°, 4.8 μs, 1 s, 3 s, 0.17 Hz, and 16, respectively. The polymer sample was dissolved in deuterated chloroform (Aldrich, 99.8 atom % deuterated) at a concentration of 50 mg/mL, and 1% v/v trimethylsilane (TMS; Aldrich) was used as the internal standard. FTIR spectra were acquired using a Nicolet 670 FTIR spectrometer with a liquid nitrogen cooled MCT detector.[46] Data were collected in transmission mode using 3 cm diameter KBr disks. Data collection consisted of 128 scans per spectrum with a resolution of 4 cm^{-1}. The collection time for each spectrum was 69 s.

The molecular weight distribution of the synthesized PLEOF was measured by gel permeation chromatography (GPC). Measurements were carried out with a Waters 717 Plus Autosampler GPC system (Waters, Milford, MA) connected to a model 616 HPLC pump, model 600S controller, and model 410 refractive index detector. The columns consisted of a styragel HT guard column (7.8 mm × 300 mm, Waters) in series with a styragel HR 4E column (7.8 mm × 300 mm, Waters) heated to 37 °C in a column heater. The GPC unit was operated using a multisystem millennium operating system, and the Empower software was used for data analysis and determination of the number ($\overline{M}_n$) and weight ($\overline{M}_w$) average molecular weights and polydispersity index (PI). The sample (20 μL), with a concentration of 10 mg/mL in tetrahydrofuran (THF; Aldrich), was eluted with degassed THF at a flow rate of 1 mL/min. Monodisperse polystyrene standards (Waters), with peak molecular weights (M_p) of 0.58−19.9, 66.35, and 143.4 kDa and polydispersities of less than 1.1, were used to construct the calibration curve.

The peptide crosslinker was purified by preparative HPLC on a 250 mm × 10 mm, 10 μm Xterra Prep RP18 column (Waters, Milford, MA) at a flow rate of 2 mL/min using a gradient 5% MeCN and 95% 0.1% aqueous TFA solvent mixture. A photodiode array detector (model 996, Waters) was used for detection at a wavelength of 214 nm. The characterization of the purified crosslinker was performed with a Fannigan 4500 Electro Spray Ionization (ESI) spectrometer (Thermo Electron, Waltham, MA).

Synthesis of PLEOF. The rate of in situ gelation of the polymerizing mixture depends on the density of unsaturated groups in the macromer, which can be maximized by using ultralow molecular weight PLA (ULMW PLA). ULMW PLA was synthesized by ring opening polymerization of the L-lactide monomer (LA), as described with slight modifications.[47] DEG and TOC were used as the bifunctional initiator and polymerization catalyst, respectively. LA was dried under vacuum at 40 °C for at least 12 h before the reaction. Briefly, 90 g of LA was heated gradually to 130 °C in a three-necked reaction flask under dry nitrogen atmosphere in an oil bath. After melting, 4.5 mL of DEG and 5 mL of TOC were added to the flask with stirring. The polymerization reaction was continued for 12 h at 140 °C with stirring. The resulting polymer mixture was dissolved in DCM and precipitated in ice-cold ether to remove the high molecular weight fraction. Next, the ether was removed by rotary evaporation; the polymer was redissolved in DCM and precipitated twice in hexane. The precipitate was dried in a vacuum (<5 mmHg) at 40 °C for at least 12 h and stored in a dry atmosphere. The synthesized ULMW PLA was characterized by ^{1}H NMR and GPC.

PLEOF was synthesized by condensation polymerization of ULMW PLA and PEG with FuCl. A schematic diagram describing the PLEOF synthesis is illustrated in Figure 1. TEA was used to neutralize the HCl produced during the reaction.[48,49] FuCl was distilled at 161 °C. The weight ratio of PEG to ULMW PLA was varied from 90:10 to 70:30 to produce a hydrophilic water soluble terpolymer. The molar

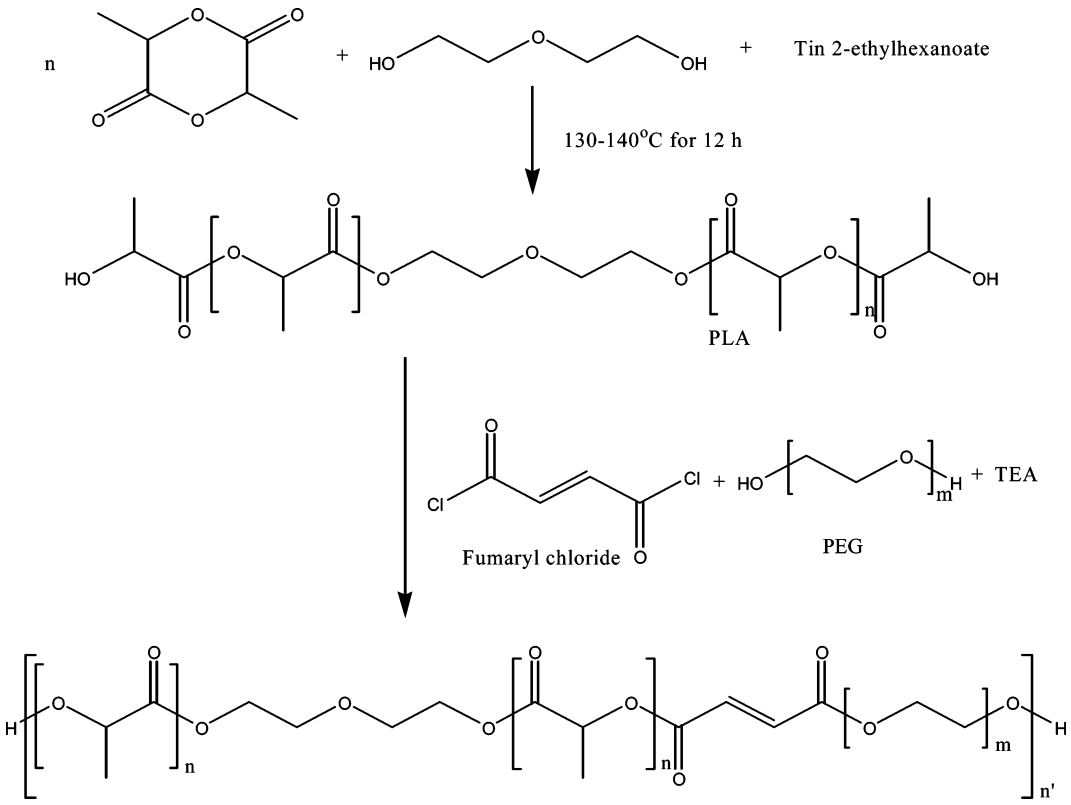

Figure 1. Schematic diagram describing synthesis of the PLEOF macromer.

ratio of FuCl/(PEG + PLA) and TEA/(PEG + PLA) was 0.9:1.0 and 1.8:1.0, respectively. In a typical reaction, 18 g of PEG and 2.0 g of ULMW PLA were dried by azeotropic distillation with toluene. The dried polymers were dissolved in 150 mL of DCM in a three-necked reaction flask under dry nitrogen atmosphere. The mixture was placed in an ice bath. After cooling to 5 °C, 0.61 mL of FuCl and 1.55 mL of TEA, each dissolved in 30 mL of DCM, were added dropwise to the reaction with stirring. The reaction was allowed to run for 6 h at 5 °C and continued for an additional 12 h under ambient conditions. After completion of the reaction, solvent was removed by rotary evaporation, and residue was dissolved in 100 mL of anhydrous ethyl acetate to precipitate the byproduct triethylamine hydrochloride. The salt was removed by filtration. Ethyl acetate was removed by rotary evaporation, and the product was dissolved in DCM and precipitated twice in cold ether. The product was dried in vacuum (<5 mmHg) at ambient temperature for at least 12 h and stored at −20 °C until used. The structure of the PLEOF macromer was characterized by ^{1}H NMR and GPC.

Synthesis of Peptide Crosslinker. A schematic diagram describing the synthesis of the peptide crosslinker is illustrated in Figure 2. The peptide sequence QPQGLAK was synthesized manually on the Rink Amide NovaGel resin (0.62 mmol/g).[50,51] A total of 100 mg of the resin was swelled in DMF for 30 min and then drained. The Fmoc protected amino acid derivative (1 equiv) and HOBt (2 equiv) were dissolved in dry DMF (3 mL), and DIC (1.1 equiv) was added to the mixture. The resulting mixture was agitated for 5−10 min and added to the resin. Next, 0.2 mL of 0.05 M DMAP was added, and the mixture was shaken for 4−6 h at 30 °C in an orbital shaker. A small amount of resin was removed and tested for the presence of unreacted amines using the Kaiser reagent. If the test result was positive, the resin was washed with DMF (5 × 3 mL), and the coupling reaction was repeated until a negative result was obtained. If the test result was negative, the resin was washed thoroughly with DMF (5 × 3 mL), treated with 20% piperidine in DMF for 2 × 15 min, and washed with DMF. The subsequent amino acids were coupled using the same method. After coupling the last amino acid of the sequence, the resin was washed with 5 × DMF and 5 × DCM. Selective deprotection of the −Mtt and −Trt protecting groups of the lysine and glutamine residues, respectively, was done by treating the peptidyl resin with 3 mL of TFA/DCM (1:99). The mixture was agitated for 2 min, filtered, and resuspended in the same volume of TFA/DCM (1:99). The procedure was repeated 7 times to ensure complete removal of −Mtt and −Trt protecting groups. After deprotection, the resin was washed with DCM (3 × 3 mL) and DMF (3 × 3 mL). The Fmoc protecting group of the glutamine residue was selectively deprotected with 20% piperidine in DMF for 2 × 15 min. The resin was washed thoroughly with DMF (5 × 3 mL) after deprotection.

The synthesized peptide was functionalized with reactive acrylate end-groups directly on the peptidyl resin by coupling acrylic acid to the amine groups of the glutamine and lysine residues. Acrylic acid (2 equiv) and HOBt (4 equiv) were dissolved in dry DMF (3 mL), and DIC (2.2 equiv) was added to the mixture. The resulting mixture was shaken for 5−10 min, added to the resin, and shaken for 4−6 h at 30 °C on an orbital shaker. The previous coupling reaction was repeated once more. A small amount of the resin was removed for the Kaiser test. If the test result was positive, the resin was washed with DMF (5 × 3 mL), and the acrylic acid coupling reaction was repeated until a negative result was obtained. If the test result was negative, the resin was washed thoroughly with DMF (5 × 3 mL) and DCM (3 × 3 mL). Next, the resin was treated with 95% TFA/2.5% TIPS/2.5% water for 2 h to cleave the peptide crosslinker from the resin. The mixture was poured into cold ether and kept at −20 °C for 24 h to precipitate the product. The suspension was centrifuged, the supernatant was decanted, and the solid was freeze-dried. The product was further purified by preparative HPLC. The HPLC fraction was lyophilized using a freeze-dryer. The product was characterized with a Fannigan 4500 ESI mass spectrometer.

Hydrogel Preparation. A schematic diagram describing the process for hydrogel formation is shown in Figure 3. Hydrogels were prepared by polymerization of the PLEOF macromer with the peptide crosslinker in aqueous solution and a redox initiation system. The redox system consisted of APS and TMEDA with equimolar concentrations of 0.02 M. Equimolar concentrations of initiator and accelerator were used to keep the pH of the polymerization mixture constant at 7.4. Hydrogels

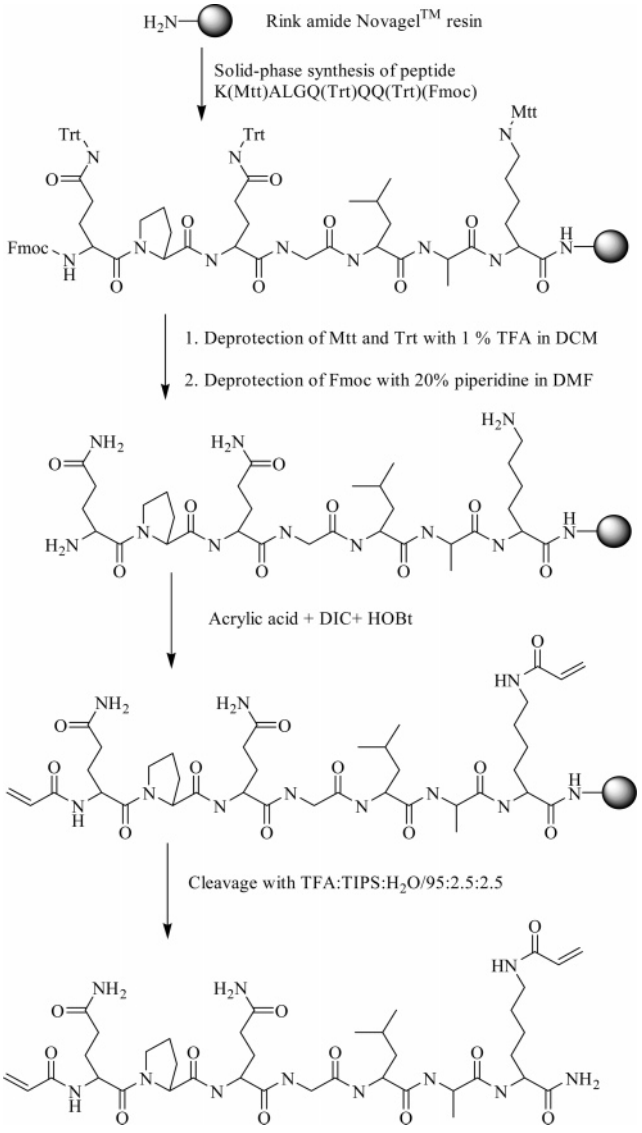

Figure 2. Schematic diagram describing synthesis of the peptide crosslinker.

crosslinked with a mutant peptide (a scrambled sequence of the same amino acids as in the peptide crosslinker, which is not degradable by MMP) and the BISAM crosslinker (non-degradable) were used as the negative control. Briefly, 30 mg of the crosslinker was dissolved in 0.825 mL of PBS by heating the mixture to 50 °C. Next, 315 mg of PLEOF macromer was added, and the mixture was heated to aid dissolution. The mixture was vortexed periodically to completely dissolve the macromer. Then, 105 μL of APS and TMEDA (0−0.2 M concentration) was added to initiate the polymerization. The mixture was mixed vigorously for 20 s and degassed, transferred into a Teflon mold (1 mm thickness), covered with a glass plate, and fastened with clips. The assembly was wrapped in aluminum foil to minimize water loss from the hydrogels by evaporation. The assembly was placed in a convection oven at 37 °C for 15 min to crosslink. After crosslinking, the gel was removed from the glass plate, and disks were cut from the gel using a 15 mm cork-borer. The disk shaped samples were used for swelling, degradation, and cell viability studies.

Hydrogel Water Content. Equilibrium water content and sol fraction of the hydrogels were determined from swelling measurements.[52] Disks (12 mm in diameter and 750 μm in thickness) were cut from the hydrogel. To measure the sol fraction, the weight of the disks immediately after crosslinking (W_{ac}) was measured. Samples were placed in 5 mL of PBS at 37 °C for 48 h, the swelling medium was changed every 12 h, and the swollen weight was measured (W_{as}). The equilibrium weight swelling ratio of the hydrogel, Q, was determined by $Q = (W_{as} - W_{asd})/W_{asd}$, where W_{asd} was the dry weight after swelling. To measure W_{asd}, the swollen disks were placed in DDI water for at least 12 h to remove excess electrolytes and were dried at ambient conditions for 12 h. This was followed by drying in vacuum at 40 °C for 1 h. The sol fraction, S, was determined by $S = (0.25W_{ac} - W_{asd})/0.25W_{ac}$, where the factor 0.25 represents the initial solid fraction of the sample based on 30 mg of crosslinker and 315 mg of PLEOF macromer.

Gelation Kinetics. The time for onset of gelation and the evolution of elasticity was determined by rheometry at a constant temperature of 37 °C in a constant strain mode.[43] The measurements were obtained with an AR-2000 rheometer (TA Instruments, New Castle, DE) using a parallel plate geometry (20 mm diameter). The polymerizing mixture was injected on the peltier plate, and the upper geometry was lowered to a gap of 500 μm. The elapsed time between mixing/injection and the start of data collection was 30 s for all experiments. A sinusoidal shear strain profile was exerted on the sample via the upper plate, and the force required to strain the sample was monitored as a function of time. The deformation amplitude was 1% to remain within the linear region of viscoelasticity. The time sweep oscillatory shear measurements were done at constant frequency of 1 Hz for 3 h. The geometry was covered with a humidity chamber to prevent dehydration during the experiment. The storage modulus (G') and loss modulus (G'') of the samples during the gelation process are reported.

Enzymatic Degradation of the Peptide Crosslinker. The enzymatic degradation of the peptide crosslinker was tested with MMP-13 using the ninhydrin reagent (Sigma).[50,51] The sample was incubated in different concentrations of MMP-13 solution (CLS-3 collagenase type 3; Worthington Biochemical, Lakewood, NJ; 150 units/mg dw) ranging from 5 to 200 μg/mL for different time periods ranging from 1 to 5 days. Briefly, 100 μL of 0.23 mg/mL peptide or mutant peptide crosslinker was added to 100 μL of 100 μg/mL MMP-13 solution in sterile PBS. In addition, the following 200 μL solutions in sterile PBS were used as controls: 0.23 mg/mL peptide crosslinker solution without MMP-13, 0.23 mg/mL mutant peptide crosslinker solution without MMP-13, and 100 μg/mL MMP-13 without peptide crosslinker. Solutions were incubated at 37 °C for 12 h, and then 50 μL of ninhydrin reagent was added to each solution. The cleavage of crosslinks by MMP-13 changed the solution color from light orange to dark blue, which was quantified by measuring the sample absorbance at 570 nm with a plate reader (Synergy HT, Bio-Tek). The measured absorbance was related to the concentration of cleaved crosslinks using a calibration curve constructed from the absorbance of solutions with known concentrations of the cleaved peptide.

Enzymatic Degradation of Peptide Crosslinked Hydrogel. Hydrogel disks were sterilized in excess ethanol. The degradation of the peptide crosslinked hydrogels was determined by measuring the weight loss as a function of incubation time in MMP-13 solution at 37 °C. Hydrogels crosslinked with a mutant peptide (scrambled sequence of the same amino acids as in the peptide crosslinker) and hydrogels crosslinked with BISAM (non-degradable crosslinker) were used as the controls. The initial dry weight, W_{d0}, was obtained by washing the samples with excess DDI water followed by drying at ambient conditions for 12 h and in a vacuum for 1 h. Next, samples were incubated in 3 mL of PBS containing 25 μg/mL MMP-13 at 37 °C with orbital mixing. The incubation media was changed every 3 days. At each time point, disks were removed, and the swollen weight (W_s) was measured. Next, the samples were washed in excess DDI water to remove electrolytes and MMP-13 and dried to determine the dry weight (W_d). The weight fraction remaining was calculated by the ratio of W_d to W_0.

Bone Marrow Stromal Cell Isolation. BMS cells were isolated from the bone marrow of young adult male Wistar rats.[53,54] After euthanasia, the femurs and tibias were aseptically excised from the hind limbs, cleaned of soft tissue, and washed in DMEM 5 times higher than for cell culture concentration of gentamicin sulfate (GS; Sigma; 100 μg/mL) to avoid contamination during the harvest. Plugs of marrow

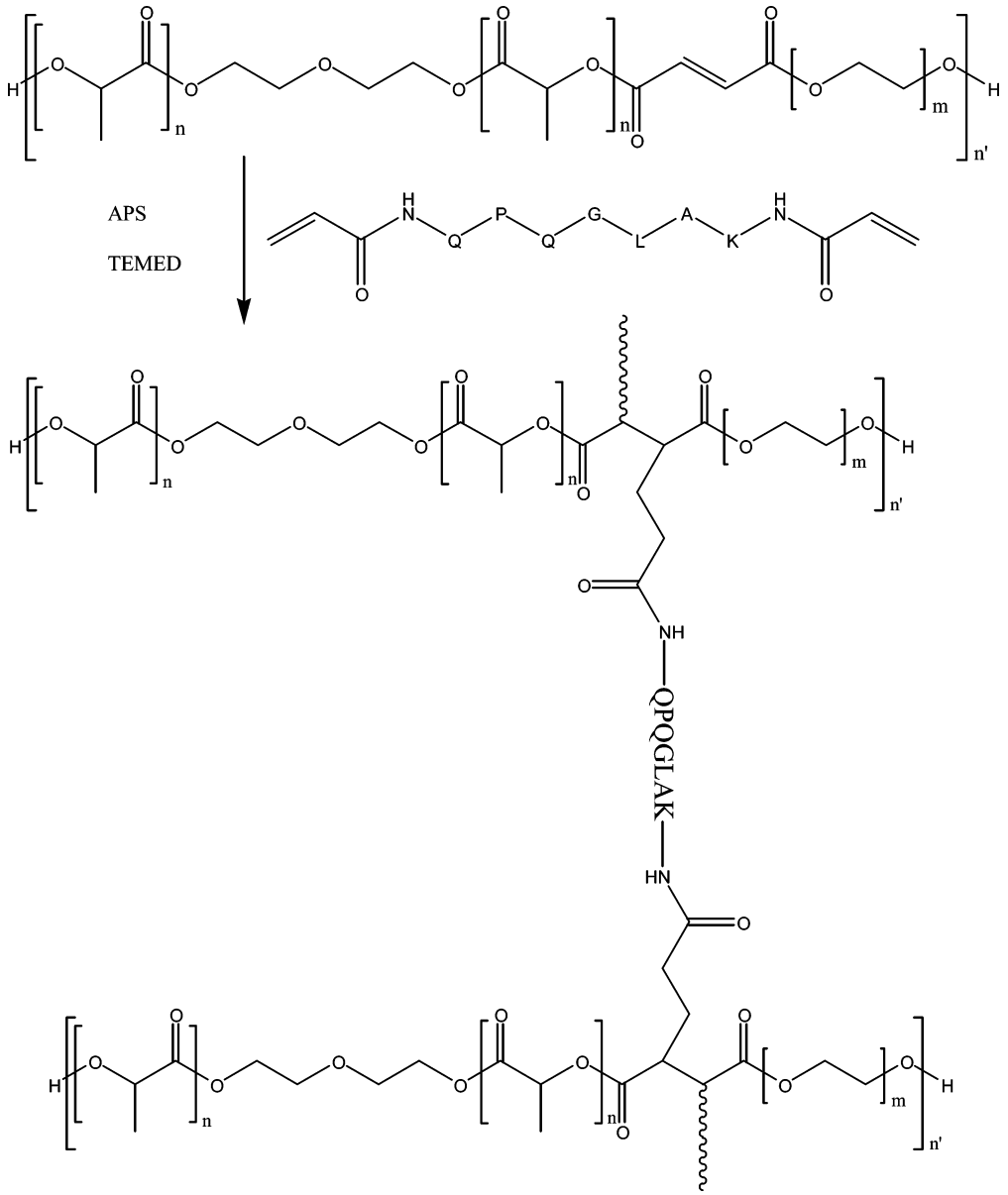

Figure 3. Schematic diagram describing the process for preparation of PLEOF hydrogel with peptide crosslinker.

were extracted by cutting the distal ends of tibias and the proximal ends of femurs to expose the marrow cavity. Small holes were bored in the other end of each bone with an 18 gauge needle. The marrow was flushed out with 5 mL of cell isolation media (DMEM supplemented with 10% FBS), 20 μg/mL fungizone (Sigma), and 50 μg/mL GS. Plugs were dispersed by shear, and the resulting suspension was collected through a 22 gauge needle to eliminate bits of bone and tissue. Cell clumps were broken up by repeatedly pipetting, and the cell suspensions were combined and centrifuged at 200*g* for 5 min. The resulting supernatant was aspirated, and cell pellets were resuspended in 12 mL of primary media and aliquoted into T-75 flasks (cells from one rat per two flasks). To prepare the primary media, 13.4 g of DMEM was dissolved in 900 mL of DDI water containing 3.7 g of sodium bicarbonate (SB), 100 mL of FBS, and 10 mL of antibiotic and antimycotic agents (1% v/v). The antibiotic and antimycotic agents included 50 μg/mL GS, 100 μg/mL streptomycin (Sigma), and 250 ng/mL fungizone. The flasks were subsequently maintained in a humidified 5% CO_2 incubator at 37 °C. Cultures were washed with PBS and replaced with fresh media at 3 and 7 days to remove hemeatopoetic and other unattached cells from the flasks. After 10 days, cells reached a sub-confluent monolayer (yielding approximately 3×10^6 cells per flask). The cells were rinsed with PBS, enzymatically lifted with 25 μL/cm^2 0.05% trypsin/0.53 mM EDTA, and centrifuged at 400*g* for 5 min. The second passage cells were used for all cell culture experiments.

Cytocompatibility of the Peptide Crosslinker. To evaluate cell viability, BMS cells were seeded in 24 well plates in primary media at a density of 4×10^4 cells/cm^2, incubated for 24 h for cell attachment, and washed with PBS. Then, the peptide crosslinker solution (36 mg/mL in PBS) was sterilized with a 0.2 μm syringe filter, and 400 μL of the solution was added to each well. After exposure for different time periods, wells were washed with PBS, and 100 μL of primary media was added to the wells and allowed to incubate for an additional 24 h to allow damaged cells to detach from the surface. A commercial cytotoxicity kit containing the fluorescence dyes calcein AM (excitation and emission wavelength of 485 and 528 nm) and ethidium homodimer-1 (EthD; Molecular Probes, Eugene, OR) (excitation and emission wavelength of 528 and 620 nm) was used to quantify the number of live and dead cells after exposure. On each plate, three wells that contained media but no cells, treated with combination dye (calcein AM and EthD), were used as controls. The numbers of live and dead cells were quantified by measuring the fluorescence of each well using a fluorescence plate reader (Synergy HT, Bio-Tek, Winooski, VT).

Cytocompatibility of the Peptide Crosslinked Hydrogel. The ASTM-F813 standard protocol was used for hydrogel cytocompatibility evaluation. In this protocol, cells are incubated in conditioned media

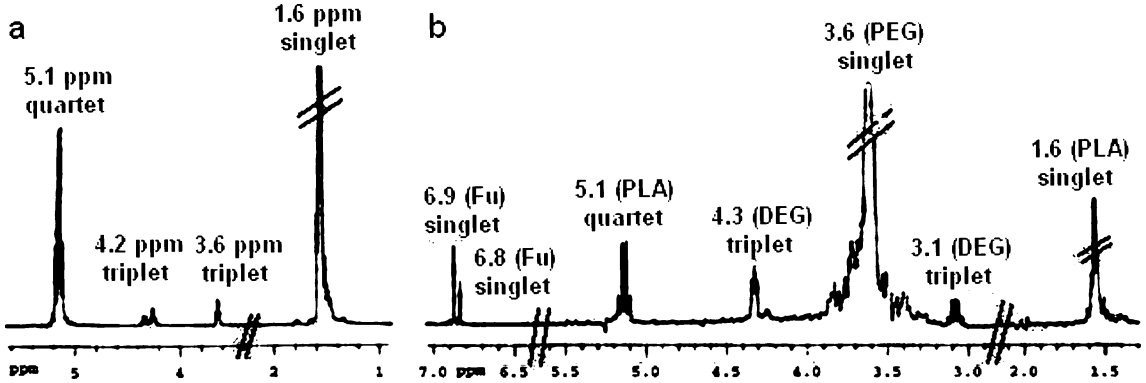

Figure 4. ^{1}H NMR spectra of ULMW PLA (a) and PLEOF (b).

for 24 h before determining the fraction of live cells. To prepare the conditioned media, 3 g of the hydrogel corresponding to 30 cm^2 of surface area was incubated in 10 mL of primary media at 37 °C for 24 h with orbital shaking to extract the sol fraction. The conditioned media was sterilized by filtration, and 100 μL was added to each well of a 96 well plate seeded with second passage BMS cells at a density of 4×10^4 cells/cm^2. After 24 h incubation, each well was washed with PBS, and 100 μL of primary media was added and incubated for an additional day. A commercial cytotoxicity kit containing the fluorescent dyes cAM and EthD was used to quantify the number of live and dead cells as described previously.

Encapsulation of BMS Cells. To sterilize PLEOF, the macromer was dissolved in anhydrous acetone (HPLC grade; Aldrich), the solution was filtered with a 0.2 μm filter (Whatman autovial syringeless filter with a PTFE membrane; Fisher), and acetone was removed by heating the solution to 50 °C in a sterile hood. The peptide crosslinker (18 mg) was dissolved in PBS (0.5 mL) and sterilized with a 0.2 μm syringe filter, and the sterile PLEOF macromer (190 mg) was added to the PBS solution. Solutions of 0.2 M APS in PBS and 0.2 M TMEDA in PBS were prepared and sterilized by 0.2 μm syringe filters. Approximately 1×10^6 BMS cells were resuspended in 100 μL of PBS and counted with a hemocytometer. Next, 65 μL of 0.2 M sterile APS and TMEDA solutions was added to the PLEOF/crosslinker mixture and vortexed. The BMS cell suspension was added to the mixture with a 200 μL pipet and mixed gently with a pre-sterilized glass rod. The mixture was injected between two sterile microscope glass slides and incubated for 10 min to crosslink. Under sterile conditions, the gel was removed from the glass slide, and the disks were cut with a sterile cork-borer. Disks were placed in a 12 well plate and incubated in 2 mL of PBS for 1 h with two PBS changes. Next, the media was changed to osteogenic media and incubated for 1 week. At each time point, disks were stained with cAM and EthD (1 μg/mL; Molecular Probes) for live and dead cell imaging. A confocal microscope (Zeiss LSM Axiovert, Berlin, Germany) was utilized to take images in the *z*-plane, perpendicular to the plane of the microscope. Depth projection micrographs were obtained from horizontal sections imaged with a depth of 5 μm.

Osteogenic Differentiation of the Encapsulated BMS Cells. BMS cells were encapsulated in the peptide crosslinked PLEOF hydrogels and cultured in osteogenic media for 21 days. At each time point, samples were removed and cut into three sections. One section was used for DNA analysis by rupturing the cell membrane by sonication to expose the DNA. The double stranded DNA (dsDNA) content of the homogenized samples was analyzed using a PicoGreen assay (Molecular Probes). Analysis of dsDNA was performed using a Synergy HT plate reader with an emission and excitation wavelength of 485 and 528 nm, respectively. Measured fluorescence intensities were correlated to cell numbers using a calibration curve constructed with BMS cells of known concentration of zero to 1×10^5 cells/mL. Another section was assayed for alkaline phosphatase (ALPase) activity using *p*-nitrophenyl phosphatase (Sigma-Aldrich). The absorbance was measured on a Synergy HT plate reader at 405 nm. The concentration

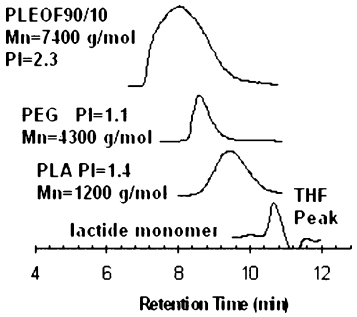

Figure 5. GPC chromatograms of L-lactide monomer, ULMW PLA, PEG, and PLEOF.

of *p*-nitrophenol was measured, correlated to ALPase activity per hour, and normalized to cell numbers, determined by DNA analysis. Standards were prepared in concentrations of 0−25 μM *p*-nitrophenol in DDI water. The last section was used to determine the calcium content using the Sigma Diagnostic Kit 587, as a measure of the total mineralized deposit in each sample. The absorbance was measured on a Synergy HT plate reader at 575 nm. Measured absorbance intensities were correlated to the amount of equivalent Ca^{2+} using a calibration curve made with calcium chloride solutions of known concentrations ranging from 0 to 100 μg/mL. Three samples were used for each time point.

Results and Discussion

PLEOF Synthesis and Characterization. The synthesized ULMW PLA was characterized by ^{1}H NMR and GPC. The ^{1}H NMR spectrum of ULMW PLA is shown in Figure 4a. In the NMR spectrum, a doublet chemical shift with a peak position at 1.6 ppm (methyl hydrogens of the lactide), two triplets with peak positions at 3.6 and 4.2 ppm (methylene hydrogens of DEG), and a quartet with a peak position at 5.1 ppm (methine hydrogen of the lactide) were observed. The M_n and PI of the ULMW PLGA were 1.2 kDa and 1.4, respectively, as shown in Figure 5. The ratio of the peaks in the NMR spectrum due to chemical shifts at 3.6 ppm (due to the hydrogens of the methylene groups attached to the ether group $-(CH_2-O-CH_2)$ of the initiator) and 5.1 ppm (due to the hydrogen attached to the methine group of the lactide repeat unit) was related to the degree of polymerization. The M_n value obtained from the ratio of the NMR peaks was consistent with the value obtained by GPC.

The structure of the PLEOF macromer was characterized by ^{1}H NMR and FTIR. The ^{1}H NMR of the PLEOF macromer is shown in Figure 4b. Four singlet chemical shifts with peak positions at 1.6, 3.5, 6.8, and 6.9 ppm, two triplets with peaks positions at 3.6 and 4.2 ppm, and a quartet with a peak position at 5.1 ppm were observed in the ^{1}H NMR spectrum of the

Table 1. Composition and Molecular Weight of the Synthesized PLEOF Macromer as a Function of the PLA/PEG Ratio

LA wt in the feed (%)	LA mol in the feed (%)	terpolymer		LA mol in the terpolymer (%)
		M_n	PI	
10	6	10630	1.81	3.7
20	13	8908	1.72	5.6
30	21	8143	1.59	6.8

terpolymer (Figure 4b). The presence of peaks at 6.90 ppm in the NMR spectrum attributable to the hydrogens of the fumarate group, and the presence of a band due to the ester carbonyl stretching vibration centered at 1725 cm^{-1} in the FTIR spectra, confirmed the incorporation of fumarate monomers into the PLEOF macromer. The ratio of the peaks in the NMR spectrum due to the chemical shifts centered at 5.1 ppm (due to the hydrogen attached to the methine group of the lactide monomer) and 3.6 ppm (due to the methylene hydrogens (-$-CH_2-$$-CH_2-$$-$O-) of the ethylene oxide repeat units) was directly related to the molar ratio of LA to EG monomers in the terpolymer. The composition and molecular weight of the PLEOF as a function of the PLA to PEG ratio is given in Table 1. For LA weight percents of 10, 20, and 30 in the feed, the feed molar percent was 6, 13, and 21, and the terpolymer molar percent was 3.7, 5.6, and 6.8, respectively. Therefore, the copolymer reactivity of PLA with fumaryl chloride was significantly less than that of PEG. The PLEOF macromer with PLA and PEG molecular weights of 1.2 kDa (PI of 1.4) and 4.3 kDa (PI of 1.1) had a M_n and PI of 7.4 kDa and 2.3, respectively, as determined by GPC (Figure 5). LA weight fractions of 0.1, 0.2, and 0.3 produced PLEOF macromers with M_n of 10.6, 8.9, and 8.1 kDa and PI of 1.8, 1.7, and 1.6, respectively, as shown in Table 1. There was a slight decrease in molecular weight and polydispersity as the percentage of the PLA was increased.

MMP-13 Degradable Peptide Crosslinker. In general, functionalization of peptides is achieved by the reaction between the amine groups of glutamine and lysine residues at the two ends of the chain with acryloyl chloride.[41] The reaction is generally carried out in solution after the cleavage of the peptide from the resin. Since the reaction is carried out in solution, the resulting product has to be dialyzed against distilled deionized water for at least 48 h to remove the unreacted compounds. Furthermore, the product yield is less than 75% because the reaction is relatively slow (24 h at ambient conditions) and produces hydrochloric acid that can attack and cleave the peptide chains even in the presence of triethylamine.[48] In this work, functionalization of the MMP-13 degradable peptide was achieved, at both N- and C-terminus, using a one step acrylic acid coupling reaction during solid phase synthesis, as shown in Figure 2. The yield of the on-bead coupling reaction was greater than 99%, which was significantly higher than the reaction of acryloyl chloride with the peptide in solution (<75%). Thus, the use of this method avoids the inherent problems of inefficient coupling for reactions in solution and eliminates the use of dialysis to remove unreacted compounds after the coupling reaction. Solid phase synthesis and functionalization were achieved for the peptide sequence Gln-Pro-Gln-Gly-Leu-Ala-Lys-NH_2 (QPQGLAK-NH_2), which matched residues 904−908 of human type II collagen with the addition of a glutamine residue to enhance solubility in aqueous solution and a lysine residue to provide an amine functional group for the acrylic acid coupling reaction.[55] This peptide sequence is cleaved by the matrix metalloproteinase-13 (MMP-13) secreted by the BMS cells.[45] The HPLC chromatograph and the ESI-MS spectrum of the purified peptide crosslinker are shown in Figure 6a,b, respectively. The molecular weight of the functionalized peptide was 847 Da. In the ESI-MS spectrum, mass numbers (m/z) 870, 848, and 425 corresponded to the monovalent sodium cation [$(M + Na)^+$] and the monovalent [$(M + H)^+$] and divalent [$(M + 2H)^{2+}$] hydrogen cations of the peptide crosslinker; mass number 666 corresponded to the hydrogen cation of the fragment produced by the cleavage of the amide bond between proline and glutamine residues [$M - QAc + H]^+$.

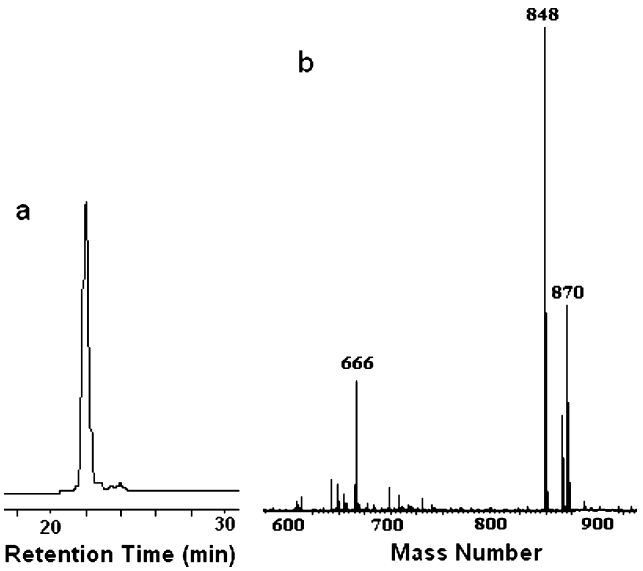

Figure 6. HPLC chromatograph and ESI mass spectrum of the purified peptide crosslinker (in the ESI spectrum: expected $(M + H)^+$ 848.24, found 848; $(M + Na)^+$ 870; $(M - QAc + H)^+$ 666).

The degradation of the peptide crosslinker was tested in the presence of MMP-13 using the ninhydrin reagent. The cleavage of the amide bond between glycine and leucine residues by MMP-13 changed the solution color from light orange to dark blue, which was quantified by measuring the sample absorbance at 570 nm. The absorbance of the MMP-13 degradable peptide and mutant peptide crosslinkers incubated with or without 40 μg/mL MMP-13 in PBS at 37 °C for 1 day is shown in Figure 7. The PBS, MMP (M), peptide (P), mutant peptide (MuP), and MMP/mutant peptide (M/MuP) solutions all showed a background absorbance in the range of 0.03−0.06 units. On the other hand, as a result of enzymatic degradation, the MMP-13/peptide crosslinker (M/P) solution showed an 8 times higher absorbance (0.32 units) as compared to that of the peptide crosslinker alone (0.04 units). These results clearly demonstrate that the peptide functionalized in the solid phase with reactive acrylate groups was enzymatically active.

Figure 8 compares the viability of BMS cells exposed to the peptide crosslinker to that of BISAM, a non-degradable crosslinker. The BMS cells were exposed to the peptide crosslinker or BISAM (30 mg of crosslinker in 825 mL of PBS) over a period of 5−30 min and then incubated for 1 day in primary media. For the peptide crosslinker, as the exposure time was increased from 5 to 15 and 30 min, the live cell fraction changed from 99 ± 2 to 97 ± 1 and 95 ± 1%, respectively; for the BISAM crosslinker, it decreased sharply from 54 ± 9 to 23 ± 5 and 17 ± 5%, respectively. BMS cell viability was drastically reduced with exposure time for BISAM but did not change with the peptide crosslinker. This is a very important result because the cytocompatibility of in situ crosslinkable hydrogels, in most cases, is limited by the exposure time of the encapsulated cells to the crosslinker. The molecular weight of

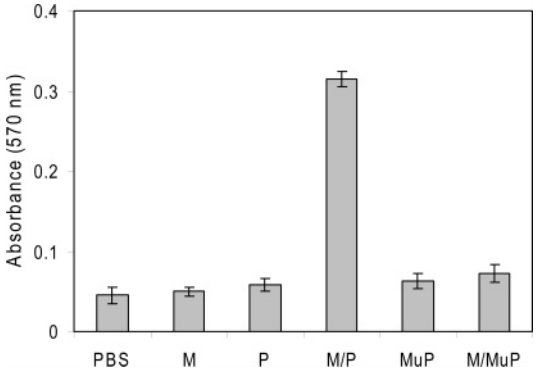

Figure 7. Comparison of the enzymatic degradation of MMP degradable and peptide crosslinkers in PBS at 37 °C after 1 day. Error bars correspond to means ± 1 SD ($n = 4$). The experimental groups included PBS without peptide or MMP-13 (PBS), MMP-13 in PBS (M), peptide crosslinker in PBS (P), peptide crosslinker with MMP-13 in PBS (M/P), mutant peptide crosslinker in PBS (MuP), and mutant peptide crosslinker with MMP-13 in PBS (M/MuP). The concentration of the peptide or mutant peptide crosslinker in PBS was 36 mg/mL, while that of MMP-13 was 40 μg/mL.

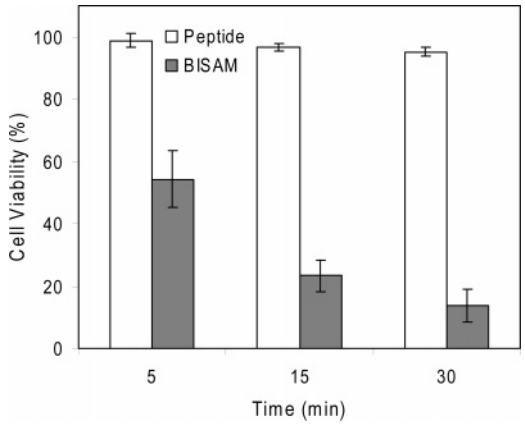

Figure 8. Viability of the BMS cells exposed to peptide and BISAM crosslinkers as a function of incubation time (36 mg/mL crosslinker concentration).

the peptide crosslinker (847 Da) is approximately 6 times higher than that of BISAM (154 Da). Therefore, the concentration of acrylate groups in the peptide and BISAM crosslinkers was 0.043 and 0.24 M, respectively. The lower concentration of cytotoxic acrylate groups certainly contributed to the high cell viability of the peptide crosslinker with exposure time. More importantly, the higher molecular weight of the peptide crosslinker as compared to that of BISAM reduced the ability of the crosslinker to penetrate the cell membrane and enter the intracellular space. It is well-established that the cytotoxicity of a compound depends on its ability to cross the cell membrane to trigger intracellular apoptotic pathways.[56−58]

Peptide Crosslinked PLEOF Gelation Kinetics. A time sweep measurement in which the storage and loss moduli were monitored as a function of gelation time and a steady-state frequency sweep measurement at low strain amplitude were used to characterize the viscoelastic properties of the hydrogel. Figure 9a,b shows the gelation kinetics of the PLEOF hydrogel without crosslinker (WO Xlinker, solid squares), with peptide crosslinker (peptide, closed circles), and with 75:25 wt % peptide/BISAM crosslinker mixture (peptide/BISAM 75:25, open circles) for gelation times of up to 1000 and 10 000 s, respectively. In Figure 9a, the ratio of G' to G'' is plotted versus time, while in Figure 9b, G' and G'' are plotted against time. The intersection of the

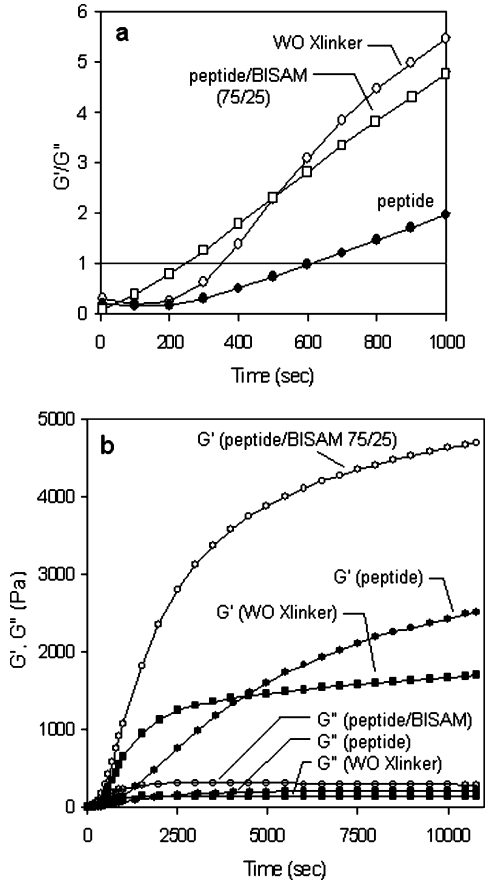

Figure 9. Storage (G') and loss (G'') moduli of the PLEOF polymerizing mixture as a function of gelation time. Experimental groups included PLEOF without crosslinker (WO Xlinker), PLEOF with peptide crosslinker (peptide), and PLEOF with a mixture of 75:25 wt % peptide/BISAM crosslinkers (peptide/BISAM 75/25). Peptide crosslinker is enzymatically degradable by MMP-13, while BISAM is a non-degradable crosslinker. Panels a and b show the first 1000 s (to determine gelation time) and up to 3 h (to determine the ultimate modulus), respectively. Data were collected at frequency of 1 Hz and temperature of 37 °C.

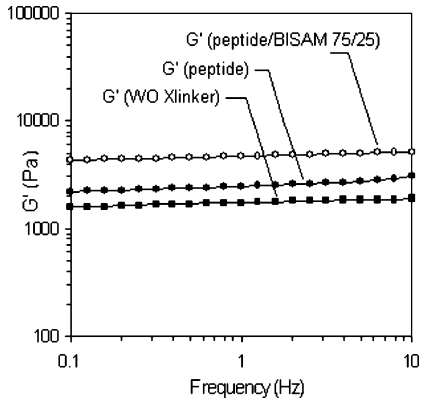

Figure 10. Storage modulus of the crosslinked PLEOF hydrogel as a function of frequency without crosslinker (WO Xlinker), with peptide crosslinker (peptide), and with 75:25 wt % peptide/BISAM crosslinkers (peptide/BISAM 75:25). Data were collected at temperature of 37 °C.

line for $G'/G'' = 1$ with the gelation curve in Figure 9a provides the gelation time of the polymerizing mixtures. The ultimate elastic shear modulus of the hydrogels can be determined from the gelation curves in Figure 9b. The frequency sweep measurements for the crosslinked hydrogels are shown in Figure 10. Frequency independence of the storage modulus confirmed that the gels were crosslinked and mechanically robust. For gelation

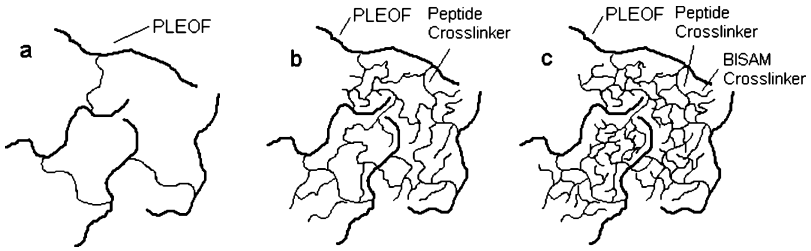

Figure 11. Schematic diagram showing the network structure of the PLEOF hydrogels without crosslinker (a), with peptide crosslinker (b), and with peptide/BISAM crosslinkers (c).

experiments in Figure 9, PLEOF without crosslinker (WO Xlinker) was used as the control group to demonstrate that the PLEOF macromer crosslinked in the absence of a crosslinking agent but that the ultimate shear modulus was relatively low (1.7 kPa according to Figure 9b). PLEOF crosslinked with 75:25 wt % peptide/BISAM crosslinkers was also used as a control group to show that the addition of a small molecular weight crosslinker (BISAM) can dramatically increase the ultimate shear modulus (4.7 kPa) while retaining enzymatic degradability of the hydrogel.

On the basis of Figure 9a, the gelation time of the PLEOF hydrogel without crosslinker, with peptide crosslinker, and with peptide/BISAM crosslinkers was 350, 600, and 250 s, respectively. In Figure 9b, the three samples show a transition from a viscous ($G'' > G'$) to an elastic response ($G' > G''$) after the gel point. While the gelation time of the peptide crosslinked PLEOF was less than that without crosslinker, the ultimate modulus (after 3 h) of the peptide crosslinked PLEOF was twice that of PLEOF without crosslinker but 50% of the PLEOF crosslinked with the peptide/BISAM mixture.

The PLEOF macromer has 2.1 reactive fumarate groups per chain (M_n = 7.4 kDa and molecular weight of the repeat unit = 3.5 kDa) and can crosslink to form a hydrogel in the absence of a crosslinking agent (gelation time of 350 s in Figure 9a), but the ultimate shear modulus of the hydrogel is relatively low (1.7 kPa in Figure 9b). Schematic diagrams in Figure 11 show the network structure of the hydrogels without crosslinker (a), with peptide crosslinker (b), and with peptide/BSIAM crosslinker mixture (c), respectively. The molecular weight of the PLEOF macromer, peptide crosslinker, and BISAM are 7.4 kDa and 845 and 154 Da, respectively. In the absence of crosslinker, the primary radicals, formed by dissociation of the initiator, react directly with the fumarate double bonds to form a PLEOF macro-radical. These macro-radicals react with fumarate groups on other PLEOF chains to form a crosslinked gel. In the absence of crosslinker, due to slow diffusivity of the macro-radicals, the crosslinking reaction becomes diffusion controlled, resulting in the formation of a network with a relatively low density of crosslinks as shown in Figure 11a. In the presence of a crosslinker, due to the lower molecular weight and higher diffusivity of the crosslinker molecules, the primary radicals react with acrylate double bonds of the crosslinker, forming a peptide or BISAM chain radical. These chain radicals react with the fumarate double bonds of the PLEOF chains to form macro-radicals that react with other macro-radicals to form a crosslinked gel. Therefore, in the presence of a crosslinker, the PLEOF chains are linked indirectly through the peptide or BISAM chain radicals (Figure 11b,c). These results demonstrate that the addition of a small fraction of a low molecular weight crosslinker, like BISM with a higher diffusivity and higher apparent reactivity, can significantly increase the ultimate shear modulus while retaining enzymatic degradability of the hydrogel by the peptide crosslinker.

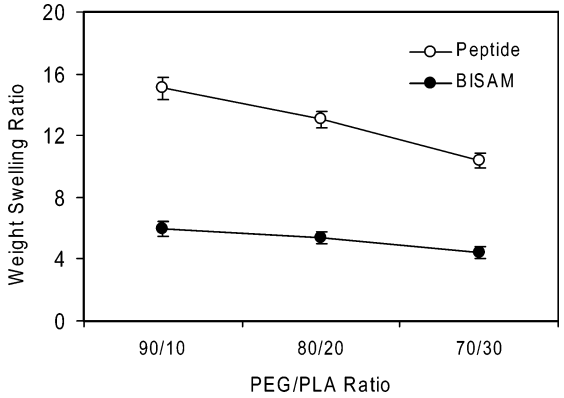

Figure 12. Comparison of equilibrium swelling ratios of the peptide and BISAM crosslinked PLEOF hydrogels as a function of PEG/PLA ratio. Error bars correspond to means ± 1 SD (n = 4).

The lower ultimate modulus of the peptide crosslinked PLEOF as compared to that of BISAM is due to slower diffusivity (higher molecular weight) of the peptide chain radicals and cannot be attributed to inefficient functionalization of the peptide crosslinker. The peptide crosslinker was functionalized in the solid phase (see Figure 2). The peptide sequence QPQGLAK was synthesized manually on the resin with the Fmoc protected amino acid derivative. After each reaction step, a small amount of resin was removed and tested for the presence of unreacted amines (on the resin) using the Kaiser reagent. If the test result was positive, the coupling reaction was repeated until a negative result was obtained. According to Kaiser et al.,[59] if the test result is negative, the coupling yield is greater than 99.4%. After seven steps, the yield of the seven-mer peptide was at least 96.5%. Next, the peptide was functionalized on the resin by coupling the amine end-groups with acrylic acid. After the coupling reaction, similar to the coupling of amino acids, a small amount of the resin was tested using the Kaiser reagent. If the result was positive, the acrylic acid coupling reaction was repeated until a negative result was obtained. After the functionalization step, the yield of the functionalized peptide with the seven-mer amino acid peptide was at least 96.5 × 0.994 = 96%. Furthermore, the peptide crosslinker was cleaved from the resin and purified by HPLC, and only the fraction eluting at 22 min was used (see Figure 6a). The mass spectral analysis (see Figure 6b) confirmed that the fraction eluting at 22 min was the seven-mer peptide with two acrylate end-groups (mass numbers 870, 848, and 425). Therefore, the purity of the peptide crosslinker was at least 96%.

Characterization of the Peptide Crosslinked PLEOF Hydrogel. The swelling ratio of the peptide crosslinked gels is compared with that of BISAM gels in Figure 12. The swelling ratio of the peptide crosslinked gels was higher than that of BISAM gels. For peptide crosslinked gels with a PLA/PEG ratio of 90:10, 80:20, and 70:30, the gel water content was 94, 93, and 91%, respectively; for BISAM gels, the water content was 86, 84, and 82%, respectively. The molar concentration of the

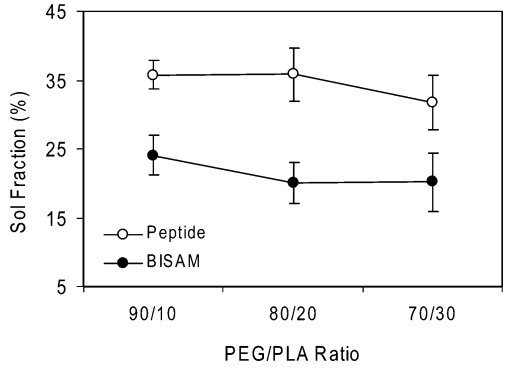

Figure 13. Comparison of sol fraction of the peptide and BISAM crosslinked PLEOF hydrogels as a function of PEG/PLA ratio. Error bars correspond to means ± 1 SD ($n = 4$).

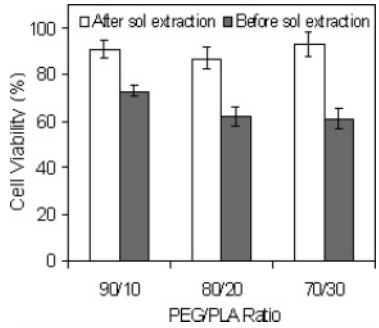

Figure 14. Viability of BMS cells after exposure to the conditioned media as a function of PEG/PLA ratio before and after extraction of the sol fraction of the hydrogels. Error bars correspond to means ± 1 SD ($n = 4$).

reactive acrylate groups in the BISAM crosslinker was 5.6 times (0.24 M for BISAM versus 0.043 M for peptide) greater than the peptide crosslinker because of the higher molecular weight of the peptide crosslinker as compared to BISAM. Therefore, the higher swelling ratio of the peptide crosslinked gels can be explained by their lower crosslink density as compared to BISAM gels. The sol fraction of the peptide and BISAM crosslinked hydrogels as a function of the PEG/PLA ratio is shown in Figure 13. The sol fraction of the peptide crosslinked gels was significantly higher than that of BISAM gels for all PEG/PLA ratios. This can be attributed to the higher molecular weight of the peptide crosslinker as compared to BISAM (845 Da for peptide vs 154 Da for BISAM) with lower diffusivity and apparent reactivity in the hydrogel matrix, which reduced the rate of incorporation of reactive chains to the gel network. Results in Figures 12 and 13 demonstrate that a relatively high molecular weight peptide crosslinker can significantly affect the water content and the rate of crosslinking (e.g., sol vs gel fraction) but that the water content can be adjusted by varying the ratio of the hydrophilic (PEG) and hydrophobic domains of the PLEOF macromer.

Cytocompatibility of the PLEOF hydrogel was tested by incubating the BMS cells in conditioned media. The conditioned media was prepared by incubating the hydrogel disks in primary media for 24 h. Figure 14 shows the viability of the BMS cells after exposure to the conditioned media as a function of the PEG/PLA ratio before and after extraction of the sol fraction of the hydrogels. For "before the sol extraction", samples were incubated in primary media for 24 h immediately after crosslinking (15 min at 37 °C); for "after the sol extraction", samples were washed in PBS for 1 h with two PBS changes before being incubated in primary media for 24 h. The before and after sol extraction samples tested the cytocompatibility of the sol/gel

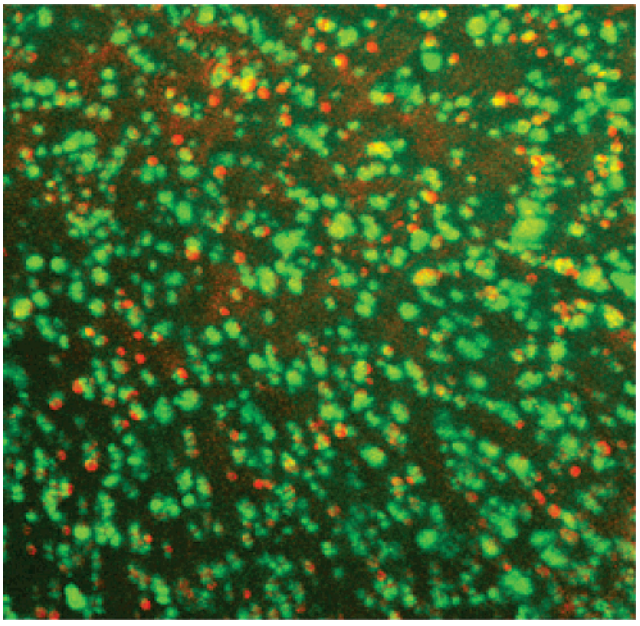

Figure 15. Live (green) and dead (red) projection images of the BMS cells after 1 week encapsulation in peptide crosslinked PLEOF hydrogel (70:30 PEG/PLA ratio). The encapsulated cells were cultured in osteogenic media. The projection image is a stack of 100 sections of the hydrogel, with a depth of 5 μm each and cross-section of 875 μm × 875 μm.

and gel fractions of the PLEOF hydrogel, respectively. For PEG/PLA ratios of 90:10, 80:20, and 70:30, cell viability after sol extraction was 91 ± 4, 87 ± 5, and 93 ± 5%, respectively; cell viability before sol extraction was 73 ± 3, 62 ± 4, and 61 ± 4%, respectively. These results demonstrate that the crosslinked fraction of the PLEOF network is not toxic to BMS cells (90% cell viability) but that the unreacted species and the growing radicals chains in the sol fraction have a significant toxic effect (av. 65% cell viability).

BMS cells were encapsulated in the peptide crosslinked PLEOF (70:30 PEG/PLA ratio) hydrogel. After 1 week, hydrogel disks were stained with cAM and EthD to image live and dead cells, respectively, with a fluorescent confocal microscope. The projection image in Figure 15 is a stack of 100 sections of the hydrogel, with a depth of 5 μm each, and a cross-section of 875 μm × 875 μm. Live (green) and dead (red) BMS cells can be seen in the image. Four images were used to estimate the fraction of live cells. Each image was divided into smaller square areas (175 μm × 175 μm), and the number of cells was counted and averaged. Cells stained only with calcein AM (green) were counted as live cells, while those that stained only with PI (red) or stained with PI and cAM (red−green cells with compromised cell membrane) were counted as dead cells. The number of live and dead cells was 377 ± 45 and 56 ± 15, respectively, corresponding to $9.2 \times 10^5 \pm 1.1$ and $1.5 \times 10^5 \pm 0.4$ cell/mL, respectively. Therefore, based on the images, the percentage of live cells was 84%. The total cell density was 1.07×10^6 cells/mL, which corresponded well with the seeded cell density, 1.07×10^6 cells/mL. The seeded density was calculated by dividing the number of seeded cells (1.0×10^6 cells) by the gel volume [0.1 mL of BMS buffer + 0.018 mL of peptide crosslinker + 0.5 mL of PBS + 0.19 mL of PLEOF + 0.065 mL of APS solution + 0.065 mL of TMEDA solution = 1.07×10^6 cells/mL]. All of the seeded cells were recovered as encapsulated cells in the gel, and approximately 84% of the encapsulated cells was viable after 1 week of incubation in osteogenic media.

Viability of the encapsulated BMS cells is certainly limited by mass transport issues, especially for systems with thicknesses greater than 1 mm.[60] Since the thickness of the hydrogel/cell constructs in our study was approximately 1 mm, mass transport limitations (oxygen, nutrients, and growth factors) can affect cell viability. Other investigators have demonstrated that cell metabolism and morphology are affected by the crosslink density of the hydrogel network.[61,62] We believe that the limited ability of the encapsulated cells to change their morphology also plays a role in their viability. Cell viability and function involves cell−cell interactions within the matrix, which necessitate changes in cell morphology to establish contact with other cellular components. The volume in the gel within which the cell is trapped changes its configuration by rearrangements of the ensemble of the surrounding chains.[63] The time constant and amplitude of these fluctuations are of the order of 200 μs and 30 nm, respectively.[64] Even at 95% water content, the hydrogel network is relatively rigid such that the time scale of the fluctuations is only a few hundreds of microseconds. These fluctuations are smaller than the time constant and amplitude required for cytoskeletal rearrangement, which is of the order of minutes and micrometers, respectively.[65] As a result, cytoskeletal rearrangement was hindered, and cell survival was reduced. Hydrogels that can degrade locally by enzymes secreted by encapsulated cells can improve cell viability by providing additional space for cytoskeletal rearrangement and morphological changes with degradation.

Differentiation of the BMS cells encapsulated in the peptide crosslinked PLEOF hydrogel was investigated by measuring ALPase activity and mineralization after incubation in osteogenic media for 21 days. As the incubation time was increased from 3 to 7 and 14 days, the ALPase activity increased from 0.80 ± 0.07 to 1.60 ± 0.80 and 4.60 ± 1.90 μM/h per cell, respectively. The extent of mineralization, measured by the amount of calcium in each sample, increased from $1.4 \times 10^{-3} \pm 1.0 \times 10^{-3}$ to $7.8 \times 10^{-2} \pm 4.0 \times 10^{-2}$ and $8.5 \times 10^{-2} \pm 5.0 \times 10^{-2}$ μg after 7, 14, and 21 days, respectively. These results demonstrate that the encapsulated BMS cells can differentiate into osteoblasts to produce a mineralized matrix.

The peptide sequence QPQGLAK-NH_2 is cleaved by MMP-13 secreted by the migrating BMS cells at the amide linkage between glycine and leucine. The ninhydrin reagent can be used to detect the increase in amine concentration as the amide linkage is degraded to amine and carboxylate groups in the presence of MMP-13. The PLEOF with a PEG/PLA ratio of 70:30 was used for enzymatic degradation experiments. Figure 16a,b shows the effects of MMP-13 concentration and incubation time on the increase in amine concentration (proportional to the number of crosslinks) for a PLEOF hydrogel crosslinked with a MMP degradable crosslinker. In Figure 16a, the incubation time was 3 days, and in Figure 16b, the concentration of MMP-13 was 25 μg/mL. The cleavage rate was linearly proportional to the ln(MMP-13) concentration (Figure 16a). In Figure 16b, the change in amine concentration for peptide crosslinked gel is compared with that of a 50:50 peptide/BISAM crosslinked hydrogel. The rate (slope of the amine concentration vs time in Figure 16b) of 100% peptide crosslinked hydrogel was greater than 4 times that of the 50:50% peptide/BISAM (0.03 vs 0.007 mM/day). These results demonstrate that the cleavage rate (hence the degradation rate) is strongly dependent on the concentrations of both enzyme (MMP-13) and substrate (peptide crosslinker).

The degradation of the peptide crosslinked hydrogel can also be determined by measuring mass loss as a function of

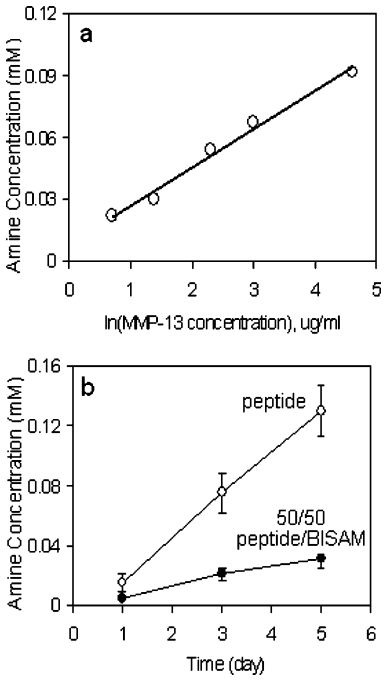

Figure 16. Effect of MMP concentration (a) and incubation time (b) on enzymatic degradation of the peptide crosslinked PLEOF hydrogels at 37 °C (70:30 PEG/PLA ratio). Incubation time in panel a was 3 days, and the MMP-13 concentration in panel b was 25 μg/mL. Error bars correspond to means ± 1 SD ($n = 4$).

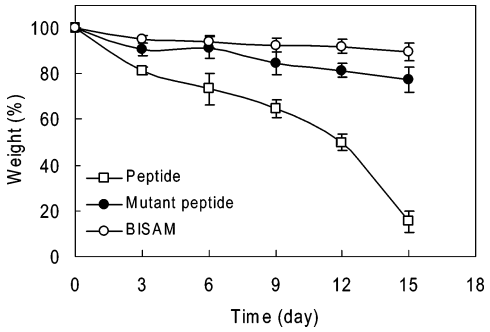

Figure 17. Dry weight of the peptide crosslinked PLEOF hydrogel (70:30 PEG/PLA ratio) as a function of incubation time in 25 μg/mL MMP-13 solution (in PBS). PLEOF hydrogels crosslinked with mutant peptide or BISAM crosslinker were used as controls. Error bars correspond to means ± 1 SD ($n = 4$).

incubation time in the MMP-13 solution. Figure 17 shows the change in dry weight of the peptide crosslinked hydrogel (70:30 PEG/PLA ratio) as a function of incubation time in PBS containing 25 μg/mL MMP-13. The change in weight of the BISAM and mutant peptide crosslinked hydrogels, as control groups, is also shown in Figure 17. PLEOF crosslinked with a mutant peptide crosslinker and BISAM crosslinker were used as control groups to demonstrate that the matrix is degraded specifically by the MMP-13 enzyme acting on its substrate, which is the peptide sequence QPQGLAK. When the scrambled amino acid sequence or a non-peptide BISAM crosslinker was used, the degradation of the matrix was significantly lower. The peptide crosslinked hydrogel lost 19, 27, 35, 50, and 85% of its dry weight after 3, 6, 9, 12, and 15 days, respectively. The BISAM and mutant peptide crosslinked hydrogels lost only 10 and 22% of their weight after 15 days. The difference in the weight loss of the BISAM and mutant peptide hydrogels was due to the difference in water content of the gels (91% for BISAM vs 82% for mutant peptide). The BISAM crosslinked hydrogel had a slow rate of mass loss by hydrolytic degradation

of the ULMW PLA blocks of the PLEOF macromer, while the peptide crosslinked hydrogel had a fast rate of mass loss by enzymatic degradation of the peptide crosslinks. The results demonstrate that the peptide crosslinked PLEOF hydrogel with tunable bimodal degradation characteristics is potentially useful as an injectable in situ crosslinkable carrier for BMS cells.

Conclusion

Injectable in situ crosslinkable biomaterials seeded with multipotent progenitor cells and coupled with minimally invasive arthroscopic techniques are an attractive alternative for treating irregularly shaped osteochondral defects. A novel in situ crosslinkable poly(lactide-*co*-ethylene oxide-*co*-fumarate) (PLEOF) macromer was developed in our laboratory with repeat units that have an excellent biocompatibility. The PLEOF macromer is produced from ultralow molecular weight poly(L-lactide) (ULMW PLA) and poly(ethylene glycol) (PEG) units linked by fumaric acid. PLA and PEG are FDA approved, and fumaric acid occurs naturally in the Kreb cycle. PLA is biodegradable, and PEG with molecular weights less than 5 kDa are excreted by the kidneys. The hydrogel water content can be adjusted by the ratio of the hydrophilic PEG to hydrophobic ULMW PLA blocks. The network density can be controlled by the density of fumarate groups and the molecular weights of the PLA and PEG blocks. The PLEOF macromer can be crosslinked with a peptide sequence like QPQGLAK with acrylate end-groups, which is degraded by MMP-13, or chemical crosslinkers like methylene bisacrylamide (BISAM) to form enzymatically or hydrolytically degradable hydrogels, respectively.

Cell viability with the peptide crosslinker as a function of exposure time was significantly higher than that with BISAM; this was attributed to the higher molecular weight of the peptide crosslinker, which reduced the ability of the crosslinker to penetrate the cell membrane and enter the intracellular space. The peptide crosslinked PLEOF hydrogel had a higher water content and higher sol fraction as compared to the BISAM crosslinked gels, which was attributed to the lower mobility and apparent reactivity of the peptide crosslinker. The relatively higher molecular weight peptide crosslinker significantly affected the water content and the rate of crosslinking (e.g., sol vs gel fraction), but the water content could be adjusted by varying the ratio of the hydrophilic (PEG) and hydrophobic domains of the PLEOF macromer. Gelation kinetic studies demonstrated that the crosslinker facilitated the process of network formation by indirectly linking the PLEOF macromers through the peptide/BISAM chain radicals and significantly increased the ultimate modulus of the hydrogel. The addition of a small fraction of a highly reactive BISAM crosslinker to the PLEOF/peptide mixture reduced gelation time and increased the shear modulus while retaining enzymatic degradability of the hydrogel. BMS cells were encapsulated in the peptide crosslinked PLEOF hydrogel; 84% of the encapsulated cells were viable after 1 week of incubation in osteogenic media. The encapsulated BMS cells differentiated to osteoblasts and produced a mineralized matrix, as measured by ALPase activity and calcium content. The BISAM crosslinked hydrogel had a slow rate of mass loss by hydrolytic degradation of the ULMW PLA blocks of the PLEOF macromer, while the peptide crosslinked hydrogel had a fast rate of mass loss by enzymatic degradation of the peptide crosslinks. The degradation rate of the hydrogel depended on the ratio of the peptide to BISAM crosslinker, MMP-13 concentration, and incubation time. The results demonstrate that the peptide crosslinked PLEOF hydrogel with tunable bimodal degradation characteristics (hydrolytic vs enzymatic) is potentially useful as an injectable in situ crosslinkable carrier for bone marrow stromal cells.

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